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Attorney Docket P1752R1  
PATENT

**CERTIFICATION UNDER 37 CFR 1.10**

**EM 168 883 307 US:** Express Mail Number **July 10, 2000:** Date of Deposit

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

*Ann Savelli*  
Ann Savelli

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION  
Assistant Commissioner of Patents  
Washington, D.C. 20231

**NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)**

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

**Antonio J. Grillo-Lopez, Rancho Santa Fe, CA**  
**Lori A. Kunkel, Oakland, CA**  
**Timothy A. Stewart, San Francisco, CA**

Title: **BLOCKING IMMUNE RESPONSE TO A FOREIGN ANTIGEN**

**1. Type of Application**

- ☐ This application is for an original, non-provisional application.
- ☒ This is a non-provisional application claiming priority to provisional application no. 60/144,405, filed July 16, 1999, the entire disclosure of which is hereby incorporated by reference.
- ☐ This is a ☐ continuation-in-part ☐ continuation ☐ divisional application claiming priority to application Serial Number     , filed     , the entire disclosure of which is hereby incorporated by reference.

**2. Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b) (Non-provisional )**

48 pages of specification  
4 pages of claims  
1 page(s) of abstract  
0 sheet(s) of drawings  
☐ formal ☐ informal

10490 U.S. PTO

09613038 071000

### 3. Declaration or Oath

*(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)*

x An unsigned declaration of the inventors is enclosed. A signed declaration of the inventors will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)

— A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)

— A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

#### 4. Assignment

*(for new and CIP applications)*

x An Assignment of the invention to GENENTECH, INC. and IDEC PHARMACEUTICALS, INC. will follow.

(for cont./div.)

\_\_\_\_ The prior application is assigned of record to Genentech, Inc.

## 5. Amendments (for continuation and divisional applications)

\_\_\_\_\_ Cancel in this application original claims \_\_\_\_ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

\_\_\_\_\_ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

**Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119**

Amend the specification by inserting before the first line the sentence:

--This is a

\_\_\_\_\_ non-provisional application

continuation

divisional

continuation-in-part

of co-pending application(s)

\_\_\_\_ Serial No. \_ filed on \_\_, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120. --

\_\_\_\_\_ International Application \_ filed on \_ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

\_\_\_\_\_ provisional application No. \_ filed \_, the entire disclosure of which is hereby incorporated by reference and to which application(s) priority is claimed under 35 USC §119.--

6. **Fee Calculation (37 CFR 1.16)**  
The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					\$690.00
Total Claims	31	- 20 =	11	X \$18.00	\$198.00
Independent Claims	5	- 3 =	2	X \$78.00	\$156.00
_____ Multiple dependent claim(s), if any				+ \$260.00	\$0.00
Filing Fee Calculation					\$1,044.00

7. **Method of Payment of Fees**  
The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of \$1,044.00. A duplicate copy of this transmittal is enclosed.

8. **Authorization to Charge Additional Fees**  
The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. A duplicate copy of this sheet is enclosed.

9. **Additional Papers Enclosed**

- ☐ Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
- ☐ Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
- ☐ A new Power of Attorney or authorization of agent.
- ☐ Other:

10. **Maintenance of Copendency of Prior Application (for continuation and divisional applications)**

*[This item **must** be completed and the necessary papers filed in the prior application if the period set in the prior application has run]*

\_\_\_\_\_ A petition, fee and/or response has been filed to extend the term in

\_\_\_\_\_ A copy of the petition for extension of time in the **prior** application is attached.

**11. Correspondence Address:**

X Address all future communications to:

GENENTECH, INC.  
Attn: Wendy Lee  
1 DNA Way  
South San Francisco, CA 94080-4990  
(650) 225-1994

Respectfully submitted,  
GENENTECH, INC.

Date: July 10, 2000

By: Wendy Lee  
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5                    **BLOCKING IMMUNE RESPONSE TO A FOREIGN ANTIGEN**

**Related Applications**

10            This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming  
priority under 35 USC 119(e) to provisional application number 60/144,405 filed July 16, 1999,  
the contents of which are incorporated herein by reference.

**Field of the Invention**

15            The present invention concerns blocking immune response to foreign antigens in a  
mammal with antagonists which bind to CD20.

**Background of the Invention**

20            Lymphocytes are one of many types of white blood cells produced in the bone marrow  
during the process of hematopoiesis. There are two major populations of lymphocytes: B  
lymphocytes (B cells) and T lymphocytes (T cells). The lymphocytes of particular interest  
herein are B cells.

25            B cells mature within the bone marrow and leave the marrow expressing an antigen-  
binding antibody on their cell surface. When a naive B cell first encounters the antigen for which  
its membrane-bound antibody is specific, the cell begins to divide rapidly and its progeny  
differentiate into memory B cells and effector cells called "plasma cells". Memory B cells have  
a longer life span and continue to express membrane-bound antibody with the same specificity as  
the original parent cell. Plasma cells do not produce membrane-bound antibody but instead  
produce the antibody in a form that can be secreted. Secreted antibodies are the major effector  
molecule of humoral immunity.

          The CD20 antigen (also called human B-lymphocyte-restricted differentiation antigen,

Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine *et al. J. Biol. Chem.* 264(19):11282-11287 (1989); and Einfeld *et al. EMBO J.* 7(3):711-717 (1988)). The antigen is also expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson *et al. Blood* 63(6):1424-1433 (1984)), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues (Tedder *et al. J. Immunol.* 135(2):973-979 (1985)). CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation (Tedder *et al., supra*) and possibly functions as a calcium ion channel (Tedder *et al. J. Cell. Biochem.* 14D:195 (1990)).

Given the expression of CD20 in B cell lymphomas, this antigen can serve as a candidate for "targeting" of such lymphomas. In essence, such targeting can be generalized as follows: antibodies specific to the CD20 surface antigen of B cells are administered to a patient. These anti-CD20 antibodies specifically bind to the CD20 antigen of (ostensibly) both normal and malignant B cells; the antibody bound to the CD20 surface antigen may lead to the destruction and depletion of neoplastic B cells. Additionally, chemical agents or radioactive labels having the potential to destroy the tumor can be conjugated to the anti-CD20 antibody such that the agent is specifically "delivered" to the neoplastic B cells. Irrespective of the approach, a primary goal is to destroy the tumor; the specific approach can be determined by the particular anti-CD20 antibody which is utilized and, thus, the available approaches to targeting the CD20 antigen can vary considerably.

The rituximab (RITUXAN®) antibody is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in US Patent No. 5,736,137 issued April 7, 1998 (Anderson *et al.*). RITUXAN® is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B cell non-Hodgkin's lymphoma. *In vitro* mechanism of action studies have demonstrated that RITUXAN® binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff *et al. Blood* 83(2):435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, RITUXAN® has been shown to have anti-proliferative effects in



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triated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD20  
antibodies do not (Maloney *et al. Blood* 88(10):637a (1996)). Synergy between RITUXAN®  
and chemotherapies and toxins has also been observed experimentally. In particular,  
RITUXAN® sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects  
of doxorubicin, CDDP, VP-16, diphtheria toxin and ricin (Demidem *et al. Cancer Chemotherapy  
& Radiopharmaceuticals* 12(3):177-186 (1997)). *In vivo* preclinical studies have shown that  
RITUXAN® depletes B cells from the peripheral blood, lymph nodes, and bone marrow of  
cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff *et al.  
Blood* 83(2):435-445 (1994)).

### Summary of the Invention

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In a first aspect, the present invention provides a method of blocking an immune response  
to a foreign antigen in a mammal, wherein the mammal is not suffering from a malignancy,  
comprising administering to the mammal a therapeutically effective amount of an antagonist  
which binds to CD20.

20  
In a further aspect, the invention provides a method of treating a mammal comprising  
administering a therapeutic agent, other than an antagonist which binds to CD20, to the mammal  
and further comprising administering an antagonist which binds to CD20 to the mammal,  
wherein the therapeutic agent is immunogenic in the mammal and the antagonist blocks an  
immune response to the therapeutic agent in the mammal.

The invention further provides a method of treating graft-versus-host or host-versus-graft  
disease in a mammal comprising administering to the mammal a therapeutically effective amount  
of an antagonist which binds to CD20.

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In addition, a method of desensitizing a mammal awaiting transplantation is provided  
which comprises administering to the mammal a therapeutically effective amount of an  
antagonist which binds to CD20.

The present invention further relates to articles of manufacture for use in the above  
methods. For example, the article of manufacture may comprise a container and a composition  
contained therein, wherein the composition comprises an antagonist which binds to CD20, and

further comprising a package insert instructing the user of the composition to treat a patient who has been or will be exposed to a foreign antigen. The article of manufacture optionally further comprises a second container and a second composition contained therein, wherein the second composition comprises a therapeutic agent.

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## **Detailed Description of the Preferred Embodiments**

### **I. Definitions**

The "CD20" antigen is a ~35 kDa, non-glycosylated phosphoprotein found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is expressed during early pre-B cell development and remains until plasma cell differentiation. CD20 is present on both normal B cells as well as malignant B cells. Other names for CD20 in the literature include "B-lymphocyte-restricted antigen" and "Bp35". The CD20 antigen is described in Clark *et al. PNAS (USA)* 82:1766 (1985), for example.

By "foreign antigen" is meant a molecule or molecules which is/are not endogenous or native to a mammal which is exposed to it. The foreign antigen may elicit an immune response, *e.g.* a humoral and/or T cell mediated response in the mammal. Generally, the foreign antigen will provoke the production of antibodies thereagainst. Examples of foreign antigens contemplated herein include immunogenic therapeutic agents, *e.g.* proteins such as antibodies, particularly antibodies comprising non-human amino acid residues (*e.g.* rodent, chimeric/humanized, and primatized antibodies); toxins (optionally conjugated to a targeting molecule such as an antibody, wherein the targeting molecule may also be immunogenic); gene therapy viral vectors, such as retroviruses and adenoviruses; grafts; infectious agents (*e.g.* bacteria and virus); alloantigens (*i.e.* an antigen that occurs in some, but not in other members of the same species) such as differences in blood types, human lymphocyte antigens (HLA), platelet antigens, antigens expressed on transplanted organs, blood components, pregnancy (Rh), and hemophilic factors (*e.g.* Factor VIII and Factor IX).

By "blocking an immune response" to a foreign antigen is meant reducing or preventing at least one immune-mediated response resulting from exposure to a foreign antigen. For example, one may dampen a humoral response to the foreign antigen, *i.e.*, by preventing or



reducing the production of antibodies directed against the antigen in the mammal. Alternatively, or additionally, one may suppress idiotype; “pacify” the removal of cells coated with alloantibody; and/or affect alloantigen presentation through depletion of antigen-presenting cells.

The mammal to be treated herein is generally one which is “not suffering from a malignancy” and hence has not been diagnosed as having a malignancy or cancer, such as B cell lymphoma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Hairy cell leukemia, chronic myeloblastic leukemia, or post-transplant lymphoproliferative disorder (PTLD).

The term “therapeutic agent” refers to a compound or composition which is used to treat a disease or disorder in a patient. The therapeutic agent may, for example, comprise a polypeptide such as an antibody; a toxin (optionally conjugated to a targeting molecule such as an antibody); a gene therapy viral vector and/or a hemophilic factor (*e.g.* Factor VIII or Factor IX). The therapeutic agent is generally administered to a mammal in a therapeutically effective amount for treating the disease or disorder of interest, wherein that amount results in an immune response being elicited to the therapeutic agent in the mammal so treated.

As used herein, “polypeptide” refers generally to peptides and proteins having more than about ten amino acids. Examples of mammalian polypeptides include molecules such as, *e.g.*, renin, a growth hormone, including human growth hormone; bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; 1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; thrombopoietin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natrietic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; a serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as brain-derived

neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF; cardiotrophins (cardiac hypertrophy factor) such as cardiotrophin-1 (CT-1); platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF- 1, TGF- 2, TGF- 3, TGF- 4, or TGF- 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; serum albumin, such as human serum albumin (HSA) or bovine serum albumin (BSA); colony stimulating factors (CSFs), *e.g.*, M-CSF, GM-CSF, and G-CSF; interleukins (ILs), *e.g.*, IL-1 to IL-10; cytokines (see below); superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; antibodies; and fragments or variants of any of the above-listed polypeptides.

The term "graft" as used herein refers to biological material derived from a donor for transplantation into a recipient. Grafts include such diverse material as, for example, isolated cells such as islet cells; tissue such as the amniotic membrane of a newborn, bone marrow, hematopoietic precursor cells, and ocular tissue, such as corneal tissue; and organs such as skin, heart, liver, spleen, pancreas, thyroid lobe, lung, kidney, tubular organs (*e.g.*, intestine, blood vessels, or esophagus), etc. The tubular organs can be used to replace damaged portions of esophagus, blood vessels, or bile duct. The skin grafts can be used not only for burns, but also as a dressing to damaged intestine or to close certain defects such as diaphragmatic hernia. The graft is derived from any mammalian source, including human, whether from cadavers or living donors. Preferably the graft is bone marrow or an organ such as heart and the donor of the graft and the host are matched for HLA class II antigens.

The term "mammalian host" as used herein refers to any compatible transplant recipient. By "compatible" is meant a mammalian host that will accept the donated graft. Preferably, the host is human. If both the donor of the graft and the host are human, they are preferably matched for HLA class II antigens so as to improve histocompatibility.

5 The term "donor" as used herein refers to the mammalian species, dead or alive, from which the graft is derived. Preferably, the donor is human. Human donors are preferably volunteer blood-related donors that are normal on physical examination and of the same major ABO blood group, because crossing major blood group barriers possibly prejudices survival of the allograft. It is, however, possible to transplant, for example, a kidney of a type O donor into an A, B or AB recipient.

10 The term "transplant" and variations thereof refers to the insertion of a graft into a host, whether the transplantation is syngeneic (where the donor and recipient are genetically identical), allogeneic (where the donor and recipient are of different genetic origins but of the same species), or xenogeneic (where the donor and recipient are from different species). Thus, in a typical scenario, the host is human and the graft is an isograft, derived from a human of the same or different genetic origins. In another scenario, the graft is derived from a species different from that into which it is transplanted, such as a baboon heart transplanted into a human recipient host, and including animals from phylogenically widely separated species, for example, a pig heart valve, or animal beta islet cells or neuronal cells transplanted into a human host.

15 By "gene therapy" is meant the general approach of introducing nucleic acid into a mammal to be treated therewith. The nucleic acid may encode a polypeptide of interest or may be antisense nucleic acid. One or more components of a gene therapy vector or composition may be immunogenic in a mammal treated therewith. For example, viral vectors (such as adenovirus, Herpes simplex I virus or retrovirus); lipids; and/or targeting molecules in the composition may induce an immune response in a mammal treated therewith.

20 The expression "desensitizing a mammal awaiting transplantation" refers to reducing or abolishing allergic sensitivity or reactivity to a transplant, prior to administration of the transplant to the mammal. This may be achieved by any mechanism, such as a reduction in anti-donor antibodies in the desensitized mammal, *e.g.* where such anti-donor antibodies are directed against human lymphocyte antigen (HLA).

25 An "autoimmune disease" herein is a non-malignant disease or disorder arising from and directed against an individual's own tissues. The autoimmune diseases herein specifically exclude malignant or cancerous diseases or conditions, especially excluding B cell lymphoma,

acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Hairy cell leukemia and chronic myeloblastic leukemia. Examples of autoimmune diseases or disorders include, but are not limited to, inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (*e.g.* atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (*e.g.* Type I diabetes mellitus or insulin dependent diabetes mellitus); multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjorgen's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia (including, but not limited to cryoglobulinemia or Coombs positive anemia) ; myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; antiphospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Behcet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia etc.

An "antagonist" is a molecule which, upon binding to CD20, destroys or depletes B cells in a mammal and/or interferes with one or more B cell functions, *e.g.* by reducing or preventing a humoral response elicited by the B cell. The antagonist preferably is able to deplete B cells (*i.e.* reduce circulating B cell levels) in a mammal treated therewith. Such depletion may be achieved via various mechanisms such antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC), inhibition of B cell proliferation and/or

induction of B cell death (*e.g.* via apoptosis). Antagonists included within the scope of the present invention include antibodies, synthetic or native sequence peptides and small molecule antagonists which bind to CD20, optionally conjugated with or fused to a cytotoxic agent. The preferred antagonist comprises an antibody.

“Antibody-dependent cell-mediated cytotoxicity” and “ADCC” refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (*e.g.* Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in a animal model such as that disclosed in Clynes *et al. PNAS (USA)* 95:652-656 (1998).

“Human effector cells” are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and carry out ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred.

The terms “Fc receptor” or “FcR” are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an “activating receptor”) and FcγRIIB (an “inhibiting receptor”), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB



contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain.  
(see Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet,  
*Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al.*, *Immunomethods* 4:25-34 (1994); and de Haas  
*et al.*, *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the  
5 future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor,  
FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J.*  
*Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)).

"Complement dependent cytotoxicity" or "CDC" refer to the ability of a molecule to lyse  
a target in the presence of complement. The complement activation pathway is initiated by the  
10 binding of the first component of the complement system (C1q) to a molecule (*e.g.* an antibody)  
complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.* as  
described in Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996), may be performed.

"Growth inhibitory" antagonists are those which prevent or reduce proliferation of a cell  
expressing an antigen to which the antagonist binds. For example, the antagonist may prevent or  
15 reduce proliferation of B cells *in vitro* and/or *in vivo*.

Antagonists which "induce apoptosis" are those which induce programmed cell death,  
*e.g.*, of a B cell, as determined by standard apoptosis assays, such as binding of annexin V,  
fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation,  
and/or formation of membrane vesicles (called apoptotic bodies).

The term "antibody" herein is used in the broadest sense and specifically covers intact  
20 monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.* bispecific  
antibodies) formed from at least two intact antibodies, and antibody fragments so long as they  
exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the  
25 antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab',  
F(ab')<sub>2</sub>, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and  
multispecific antibodies formed from antibody fragments.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons,  
composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain



is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain ( $V_H$ ) followed by a number of constant domains. Each light chain has a variable domain at one end ( $V_L$ ) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a  $\beta$ -sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the  $\beta$ -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain

variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the  $V_H$ - $V_L$  dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa ( $\kappa$ ) and lambda ( $\lambda$ ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Single-chain Fv" or "scFv" antibody fragments comprise the  $V_H$  and  $V_L$  domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the  $V_H$  and  $V_L$  domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain ( $V_H$ ) connected to a light-chain variable domain ( $V_L$ ) in the same polypeptide chain ( $V_H - V_L$ ). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another

species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). Chimeric antibodies of interest herein include “primatized” antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (*e.g.* Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences (US Pat No. 5,693,780).

“Humanized” forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

The term “hypervariable region” when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a “complementarity determining region” or “CDR” (*e.g.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a “hypervariable loop” (*e.g.* residues 26-32 (L1), 50-52

(L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

5 An antagonist "which binds" an antigen of interest, *e.g.*, CD20, is one capable of binding that antigen with sufficient affinity and/or avidity such that the antagonist is useful as a therapeutic agent for targeting a cell expressing the antigen.

10 Examples of antibodies which bind the CD20 antigen include: "C2B8" which is now called "rituximab" ("RITUXAN®") (US Patent No. 5,736,137, expressly incorporated herein by reference); the yttrium-[90]-labeled 2B8 murine antibody designated "Y2B8" (US Patent No. 5,736,137, expressly incorporated herein by reference); murine IgG2a "B1" optionally labeled with <sup>131</sup>I to generate the "<sup>131</sup>I-B1" antibody (BEXXAR™) (US Patent No. 5,595,721, expressly incorporated herein by reference); murine monoclonal antibody "1F5" (Press *et al. Blood* 69(2):584-591 (1987)); "chimeric 2H7" antibody (US Patent No. 5,677,180, expressly incorporated herein by reference); and monoclonal antibodies L27, G28-2, 93-1B3, B-C1 or NU-B2 available from the International Leukocyte Typing Workshop (Valentine *et al.*, In: *Leukocyte Typing* III (McMichael, Ed., p. 440, Oxford University Press (1987)).

15 The terms "rituximab" or "RITUXAN®" herein refer to the genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen and designated "C2B8" in US Patent No. 5,736,137, expressly incorporated herein by reference. The antibody is an IgG<sub>1</sub> kappa immunoglobulin containing murine light and heavy chain variable region sequences and human constant region sequences. Rituximab has a binding affinity for the CD20 antigen of approximately 8.0nM.

20 An "isolated" antagonist is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antagonist, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antagonist will be purified (1) to greater than 95% by weight of antagonist as determined by the Lowry method, and most preferably more than 99% by



weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antagonist includes the antagonist *in situ* within recombinant cells since at least one component of the antagonist's natural environment will not be present. Ordinarily, however, isolated antagonist will be prepared by at least one purification step.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease or disorder as well as those in which the disease or disorder is to be prevented. Hence, the mammal may have been diagnosed as having the disease or disorder or may be predisposed or susceptible to the disease.

The expression "therapeutically effective amount" refers to an amount of the antagonist which is effective for preventing, ameliorating or treating the disease or condition in question.

The term "immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to suppress or mask the immune system of the mammal being treated herein. This would include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines (see U.S. Pat. No. 4,665,077, the disclosure of which is incorporated herein by reference); antiproliferative agents, such as azathioprine, leflunomide or sirolimus; cyclophosphamide; bromocryptine; danazol; dapson; glutaraldehyde (which masks the MHC antigens, as described in U.S. Pat. No. 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as corticosteroids, *e.g.*, prednisone, methylprednisolone, and dexamethasone; mycophenolate mofetil; calcineurin inhibitors (*e.g.* tacrolimus); cytokine or cytokine receptor antagonists including anti-interferon- $\gamma$ , - $\beta$ , or - $\alpha$  antibodies, anti-tumor necrosis factor- $\alpha$  antibodies, anti-tumor necrosis factor- $\beta$  antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CD11a and anti-CD18 antibodies; anti-L3T4 antibodies; anti-lymphocyte antibodies, *e.g.*



polyclonal anti-lymphocyte antibodies; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a  
 antibodies; soluble peptide containing a LFA-3 binding domain (WO 90/08187 published  
 7/26/90); streptokinase; TGF- $\beta$ ; streptodornase; RNA or DNA from the host; FK506; RS-61443;  
 deoxyspergualin; rapamycin; T-cell receptor (Cohen *et al.*, U.S. Pat. No. 5,114,721); T-cell  
 5 receptor fragments (Offner *et al.*, *Science*, 251: 430-432 (1991); WO 90/11294; Ianeway, *Nature*,  
 341: 482 (1989); and WO 91/01133); and T cell receptor antibodies (EP 340,109) such as  
 T10B9.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents  
 the function of cells and/or causes destruction of cells. The term is intended to include  
 10 radioactive isotopes (*e.g.* At<sup>211</sup>, I<sup>131</sup>, I<sup>125</sup>, Y<sup>90</sup>, Re<sup>186</sup>, Re<sup>188</sup>, Sm<sup>153</sup>, Bi<sup>212</sup>, P<sup>32</sup> and radioactive  
 isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or  
 enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer.  
 Examples of chemotherapeutic agents include alkylating agents such as thiotepa and  
 15 cyclophosphamide (CYTOXAN<sup>TM</sup>); alkyl sulfonates such as busulfan, improsulfan and  
 piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines  
 and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramidate,  
 triethylenethiophosphoramidate and trimethylolomelamine; nitrogen mustards such as  
 chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine,  
 20 mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine,  
 trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine,  
 lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin,  
 anthramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabycin, carminomycin,  
 carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-  
 25 norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins,  
 mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin,  
 quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin;  
 anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as  
 denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-

mercaptapurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2''-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.* paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, NJ) and doxetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin;

glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- $\alpha$  and - $\beta$ ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF- $\beta$ ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- $\alpha$  and TGF- $\beta$ ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- $\alpha$ , - $\beta$ , and - $\gamma$ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-15; a tumor necrosis factor such as TNF- $\alpha$  or TNF- $\beta$ ; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs,  $\beta$ -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the antagonists disclosed herein and,

optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term “package insert” is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

## **II. Production of Antagonists**

The methods and articles of manufacture of the present invention use, or incorporate, an antagonist which binds to CD20. Accordingly, methods for generating such antagonists will be described here.

The CD20 antigen to be used for production of, or screening for, antagonist(s) may be, *e.g.*, a soluble form of the antigen or a portion thereof, containing the desired epitope. Alternatively, or additionally, cells expressing CD20 at their cell surface can be used to generate, or screen for, antagonist(s). Other forms of CD20 useful for generating antagonists will be apparent to those skilled in the art.

While the preferred antagonist is an antibody, antagonists other than antibodies are contemplated herein. For example, the antagonist may comprise a small molecule antagonist optionally fused to, or conjugated with, a cytotoxic agent (such as those described herein). Libraries of small molecules may be screened against CD20 in order to identify a small molecule which binds to that antigen. The small molecule may further be screened for its antagonistic properties and/or conjugated with a cytotoxic agent.

The antagonist may also be a peptide generated by rational design or by phage display (see, *e.g.*, WO98/35036 published 13 August 1998). In one embodiment, the molecule of choice may be a “CDR mimic” or antibody analogue designed based on the CDRs of an antibody. While such peptides may be antagonistic by themselves, the peptide may optionally be fused to a cytotoxic agent so as to add or enhance antagonistic properties of the peptide.

A description follows as to exemplary techniques for the production of the antibody antagonists used in accordance with the present invention.

(i) *Polyclonal antibodies*

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N}=\text{C}=\text{NR}$ , where R and  $\text{R}^1$  are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100  $\mu\text{g}$  or 5  $\mu\text{g}$  of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) *Monoclonal antibodies*

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).



In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization.

Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).



The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large

phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al.*, *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) *Humanized antibodies*

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-327 (1988); Verhoeyen *et al.*, *Science*, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-

fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.*, 151:2296 (1993); Chothia *et al.*, *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al.*, *J. Immunol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

(iv) *Human antibodies*

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin

gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Bruggermann *et al.*, *Year in Immuno.*, 7:33 (1993); and US Patent Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty *et al.*, *Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature*, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), or Griffith *et al.*, *EMBO J.* 12:725-734 (1993). See, also, US Patent Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by *in vitro* activated B cells (see US Patents 5,567,610 and 5,229,275).

(v) *Antibody fragments*

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.*, *Science*, 229:81 (1985)). However, these fragments can now be produced directly

by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')<sub>2</sub> fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). According to another approach, F(ab')<sub>2</sub> fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; US Patent No. 5,571,894; and US Patent No. 5,587,458. The antibody fragment may also be a "linear antibody", *e.g.*, as described in US Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) *Bispecific antibodies*

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of CD20. Alternatively, an anti-CD20 binding arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the B cell. Bispecific antibodies may also be used to localize cytotoxic agents to the B cell. These antibodies possess a CD20-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* F(ab')<sub>2</sub> bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al.*, *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the



product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al.*, *EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986).

According to another approach described in US Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C<sub>H</sub>3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of



identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

5 Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking  
10 methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies  
15 are proteolytically cleaved to generate F(ab')<sub>2</sub> fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the  
20 other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al.*, *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')<sub>2</sub>  
25 molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain ( $V_H$ ) connected to a light-chain variable domain ( $V_L$ ) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the  $V_H$  and  $V_L$  domains of one fragment are forced to pair with the complementary  $V_L$  and  $V_H$  domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al.* *J. Immunol.* 147: 60 (1991).

### III. Conjugates and Other Modifications of the Antagonist

The antagonist used in the methods or included in the articles of manufacture herein is optionally conjugated to a cytotoxic agent.

Chemotherapeutic agents useful in the generation of such antagonist-cytotoxic agent conjugates have been described above.

Conjugates of an antagonist and one or more small molecule toxins, such as a calicheamicin, a maytansine (US Patent No. 5,208,020), a trichothene, and CC1065 are also contemplated herein. In one embodiment of the invention, the antagonist is conjugated to one or more maytansine molecules (*e.g.* about 1 to about 10 maytansine molecules per antagonist molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antagonist (Chari *et al.* *Cancer Research* 52: 127-131 (1992)) to generate a maytansinoid-antagonist conjugate.

Alternatively, the antagonist is conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to,  $\gamma_1^I$ ,  $\alpha_2^I$ ,  $\alpha_3^I$ , N-acetyl- $\gamma_1^I$ , PSAG and  $\theta_1^I$  (Hinman *et al. Cancer Research* 53: 3336-3342 (1993) and Lode *et al. Cancer Research* 58: 2925-2928 (1998)).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates antagonist conjugated with a compound with nucleolytic activity (*e.g.* a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

A variety of radioactive isotopes are available for the production of radioconjugated antagonists. Examples include  $At^{211}$ ,  $I^{131}$ ,  $I^{125}$ ,  $Y^{90}$ ,  $Re^{186}$ ,  $Re^{188}$ ,  $Sm^{153}$ ,  $Bi^{212}$ ,  $P^{32}$  and radioactive isotopes of Lu.

Conjugates of the antagonist and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al. Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antagonist. See

WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari *et al. Cancer Research* 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antagonist and cytotoxic agent may be made, *e.g.* by recombinant techniques or peptide synthesis.

In yet another embodiment, the antagonist may be conjugated to a "receptor" (such as streptavidin) for utilization in tumor pretargeting wherein the antagonist-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.* avidin) which is conjugated to a cytotoxic agent (*e.g.* a radionucleotide).

The antagonists of the present invention may also be conjugated with a prodrug-activating enzyme which converts a prodrug (*e.g.* a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of such conjugates includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratiopeptidase, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as  $\beta$ -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs;  $\beta$ -lactamase useful for converting drugs derivatized with  $\beta$ -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs.

Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be

used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328: 457-458 (1987)). Antagonist-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the antagonist by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antagonist of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature*, 312: 604-608 (1984)).

Other modifications of the antagonist are contemplated herein. For example, the antagonist may be linked to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol.

The antagonists disclosed herein may also be formulated as liposomes. Liposomes containing the antagonist are prepared by methods known in the art, such as described in Epstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published October 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of an antibody of the present invention can be conjugated to the liposomes as described in Martin *et al.* *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon *et al.* *J. National Cancer Inst.* 81(19)1484 (1989).

Amino acid sequence modification(s) of protein or peptide antagonists described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antagonist. Amino acid sequence variants of the antagonist are prepared by introducing appropriate nucleotide changes into the antagonist nucleic acid, or by



peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antagonist. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antagonist, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the antagonist that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antagonist variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antagonist with an N-terminal methionyl residue or the antagonist fused to a cytotoxic polypeptide. Other insertional variants of the antagonist molecule include the fusion to the N- or C-terminus of the antagonist of an enzyme, or a polypeptide which increases the serum half-life of the antagonist.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antagonist molecule replaced by different residue. The sites of greatest interest for substitutional mutagenesis of antibody antagonists include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table



1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

**Table 1**

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr

Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antagonist are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the antagonist also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antagonist to improve its stability (particularly where the antagonist is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody. Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage display. Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants

thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or in additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antagonist alters the original glycosylation pattern of the antagonist. By altering is meant deleting one or more carbohydrate moieties found in the antagonist, and/or adding one or more glycosylation sites that are not present in the antagonist.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antagonist is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antagonist (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the antagonist are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antagonist.

It may be desirable to modify the antagonist of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antagonist. This may be achieved by introducing one or more amino acid substitutions in an Fc region of an antibody antagonist. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

To increase the serum half life of the antagonist, one may incorporate a salvage receptor binding epitope into the antagonist (especially an antibody fragment) as described in US Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, or IgG<sub>4</sub>) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

#### IV. Pharmaceutical Formulations

Therapeutic formulations of the antagonists used in accordance with the present invention are prepared for storage by mixing an antagonist having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's*

*Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Exemplary anti-CD20 antibody formulations are described in WO98/56418, expressly incorporated herein by reference. This publication describes a liquid multidose formulation comprising 40 mg/mL rituximab, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% polysorbate 20 at pH 5.0 that has a minimum shelf life of two years storage at 2-8°C. Another anti-CD20 formulation of interest comprises 10mg/mL rituximab in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7mg/mL polysorbate 80, and Sterile Water for Injection, pH 6.5.

Lyophilized formulations adapted for subcutaneous administration are described in WO97/04801. Such lyophilized formulations may be reconstituted with a suitable diluent to a high protein concentration and the reconstituted formulation may be administered subcutaneously to the mammal to be treated herein.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide a cytotoxic agent, chemotherapeutic agent, cytokine or immunosuppressive agent (*e.g.* one which acts on T

cells, such as cyclosporin or an antibody that binds T cells, *e.g.* one which binds LFA-1). The effective amount of such other agents depends on the amount of antagonist present in the formulation, the type of disease or disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and  $\gamma$  ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

## V. Treatment with the Antagonist

The antagonist which binds to CD20 may be used to block an immune response to a foreign antigen in a mammal (preferably a human), wherein the mammal is not suffering from a malignancy. Preferably, the antagonist comprises an anti-CD20 antibody. The antibody in one embodiment is not conjugated with a cytotoxic agent, in another, the antibody is conjugated with a cytotoxic agent (*e.g.* Y2B8 or <sup>131</sup>I-B1).



1 The mammal to be treated herein may be exposed to both the antagonist which binds to  
CD20 and a further different therapeutic agent, *e.g.*, where the therapeutic agent is immunogenic  
in the mammal. In this embodiment, the antagonist may block an immune response to the  
therapeutic agent in the mammal treated therewith. The therapeutic benefit may also include  
5 blocking removal of antibody coated cells by the spleen. The therapeutic agent is administered  
to the mammal in a therapeutically effective amount to treat a disease or disorder which could  
benefit from administration of the therapeutic agent. In this embodiment, one may administer  
the therapeutic agent and the antagonist essentially simultaneously or separately in either order to  
the mammal. Hence, the antagonist may be administered to the mammal prior to the therapeutic  
10 agent, or the therapeutic agent may be administered to the mammal prior to the antagonist.

The antagonist which binds to CD20 may thus be used to treat graft-versus-host or host-  
versus-graft disease in a mammal and/or to desensitize a mammal awaiting transplantation.

15 For the various indications disclosed herein, a composition comprising an antagonist  
which binds to CD20 will be formulated, dosed, and administered in a fashion consistent with  
good medical practice. Factors for consideration in this context include the particular disease or  
condition being treated, the particular mammal being treated, the clinical condition of the  
individual patient, the cause of the disease or condition, the site of delivery of the agent, the  
method of administration, the scheduling of administration, and other factors known to medical  
practitioners. The therapeutically effective amount of the antagonist to be administered will be  
20 governed by such considerations.

As a general proposition, the therapeutically effective amount of the antagonist  
administered parenterally per dose will be in the range of about 0.1 to 20 mg/kg of patient body  
weight per day, with the typical initial range of antagonist used being in the range of about 2 to  
10 mg/kg.

25 The preferred antagonist is an antibody, *e.g.* an antibody such as RITUXAN®, which is  
not conjugated to a cytotoxic agent. Suitable dosages for an unconjugated antibody are, for  
example, in the range from about 20mg/m<sup>2</sup> to about 1000mg/m<sup>2</sup>. In one embodiment, the dosage  
of the antibody differs from that presently recommended for RITUXAN®. For example, one may  
administer to the patient one or more doses of substantially less than 375mg/m<sup>2</sup> of the antibody,

*e.g.* where the dose is in the range from about 20mg/m<sup>2</sup> to about 250mg/m<sup>2</sup>, for example from about 50mg/m<sup>2</sup> to about 200mg/m<sup>2</sup>.

Moreover, one may administer one or more initial dose(s) of the antibody followed by one or more subsequent dose(s), wherein the mg/m<sup>2</sup> dose of the antibody in the subsequent dose(s) exceeds the mg/m<sup>2</sup> dose of the antibody in the initial dose(s). For example, the initial dose may be in the range from about 20mg/m<sup>2</sup> to about 250mg/m<sup>2</sup> (*e.g.* from about 50mg/m<sup>2</sup> to about 200mg/m<sup>2</sup>) and the subsequent dose may be in the range from about 250mg/m<sup>2</sup> to about 1000mg/m<sup>2</sup>.

As noted above, however, these suggested amounts of antagonist are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated above. For example, relatively higher doses may be needed initially for the treatment of ongoing and acute diseases. To obtain the most efficacious results, depending on the disease or condition, the antagonist is administered as close to the first sign, diagnosis, appearance, or occurrence of the disease or condition as possible or during remissions of the disease or condition.

The antagonist is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local immunosuppressive treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antagonist may suitably be administered by pulse infusion, *e.g.*, with declining doses of the antagonist. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

One may administer other compounds, such as cytotoxic agents, chemotherapeutic agents, immunosuppressive agents and/or cytokines with the antagonists herein. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

Aside from administration of protein antagonists to the patient the present application contemplates administration of antagonists by gene therapy. Such administration of nucleic acid

encoding the antagonist is encompassed by the expression "administering a therapeutically effective amount of an antagonist". See, for example, WO96/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; *in vivo* and *ex vivo*. For *in vivo* delivery the nucleic acid is injected directly into the patient, usually at the site where the antagonist is required. For *ex vivo* treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, *e.g.* U.S. Patent Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for *ex vivo* delivery of the gene is a retrovirus.

The currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.* capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu *et al.*, *J. Biol. Chem.* 262:4429-4432 (1987); and Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see

Anderson *et al.*, *Science* 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

## VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the diseases or conditions described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds or contains a composition which is effective for treating the disease or condition of choice and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is the antagonist which binds CD20. The label or package insert indicates that the composition is used for blocking an immune response to a foreign antigen and/or treating the various diseases or conditions as hereindescribed. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable diluent buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. In one embodiment, the second container holds or contains a composition wherein the active agent in that composition is a therapeutic agent. The package insert may indicate that the patient is to be treated with both compositions in this embodiment of the invention. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all citations in the specification are expressly incorporated herein by reference.

### Example 1

#### Blocking an Immune Response to a Therapeutic Protein

In the present example, an anti-CD20 antibody is used to block an immune response to the therapeutic protein; megakaryocyte growth and development factor (MGDF, also known as thrombopoietin or Mpl ligand). In particular, a pegylated form of recombinant human MGDF

(PEG-rHuMGDF) has been reported to develop neutralizing antibodies in cancer patients and platelet donors. Administration of an anti-CD20 antibody as disclosed herein will ameliorate an immune response, especially the humoral response, directed against PEG-rHuMGDF.

PEG-rHuMGDF is prepared as described in US Patent No. 5,795,569 issued August 18, 1998, expressly incorporated herein by reference. PEG-rHuMGDF consists of amino acids 1-163 (numbering from the beginning of the mature protein) of human *E.coli* derived MGDF with a single polyethylene glycol (PEG) attached to the  $\alpha$ -amino group at the N-terminus of the polypeptide.

MGDF is administered to patients suffering from thrombocytopenia, *e.g.* as a result of chemotherapy or radiation therapy, in dosages appropriate for increasing platelet counts in the patients; *e.g.* in the range of 0.1 to 1000 micrograms of MGDF per kilogram of body weight. MGDF therapy is optionally combined with administration of one or more additional cytokines, such as erythropoietin (EPO), interleukin-3 (IL-3) and granulocyte megakaryocyte colony stimulating factor (GM-CSF).

Anti-MGDF antibody titers in the patient so treated are monitored by a suitable assay, such as an antibody titer enzyme linked immunosorbent assay (ELISA). Those patients demonstrating a low titer immune response to MGDF are then candidates for treatment with an anti-CD20 antibody, such as RITUXAN®. The anti-CD20 antibody may be administered subsequent to, simultaneously with, or following further treatment with MGDF. A suitable dosage of the anti-CD20 antibody is 375mg/m<sup>2</sup> by four weekly infusions. However, lesser doses, *e.g.*, in the range from about 50 to about 250mg/m<sup>2</sup> may also be administered. Administration of the anti-CD20 antibody to the patient will prevent, or reduce to an acceptable level, the formation of anti-MGDF antibodies in patients treated with both MGDF and anti-CD20 as described above.

Hence, for a protein drug of great therapeutic value and known immunogenicity, co-administration of an anti-CD20 antibody as herein-described will treat the immunogenic side-effect(s) associated with administration of that protein drug to a patient.

## **Example 2**

### **Blocking an Immune Response to a Gene Therapy Viral Vector**



E1, E3-deleted, replication-deficient recombinant adenoviruses have been evaluated for their capability to transfer therapeutic genes *in vivo*. New vectors with additional deletions in the E2a or the E4 regions have been developed. Christ *et al. Immunol. Let.* 57:19-25 (1997). Despite the deletion of these viral regions, low levels of early and late viral genes are expressed *in vivo*. Production of anti-adenovirus antibodies, the cellular immune response as well as the early non-specific clearance of the vectors constitute barriers to successful gene therapy. In order to inhibit, or reduce to an acceptable level, the production of neutralizing antibodies to adenovirus, an anti-CD20 antibody (*e.g.* RITUXAN®) is administered to the gene therapy patient as herein described.

For example, cystic fibrosis patients are treated with a replication-deficient adenovirus expressing the human cystic fibrosis transmembrane conductance regulator (CFTR) (Bellon *et al. Human Gene Therapy* 8:15-25 (1997)). Suitable dosages of the CFTR gene therapy vector (defined in terms of viral plaque forming units, pfu) are administered via aerosolization in order to achieve expression of CFTR in the lungs (*e.g.* from about  $10^7$  to about  $10^9$  pfu). Anti-adenovirus antibodies in the patient may be detected by ELISA, immunofluorescence, and/or complement fixation. In those patients demonstrating anti-adenovirus antibodies, an anti-CD20 antibody (*e.g.* chimeric 2H7; US Patent No. 5,677,180), optionally in combination with other immunosuppressive drugs (*e.g.* cyclophosphamide, FK506, or monoclonal antibodies that block either the T cell receptor or costimulation pathways), is administered to the patient prior to, simultaneously with, or following re-administration of the gene therapy vector. A suitable dosage of the anti-CD20 antibody is 375mg/m<sup>2</sup> by four weekly infusions. Administration of the anti-CD20 antibody will reduce or eliminate an immune response in the patients (*e.g.* by reducing anti-adenovirus antibody production), and thereby facilitate successful gene therapy retreatments.

### **Example 3**

#### **Blocking an Immune Response to a Transplant**

An anti-CD20 antibody is used as part of combination immunosuppressive regimens for prophylaxis of acute rejection. In this setting, an anti-CD20 antibody, such as RITUXAN®, is administered in the peri-transplant period as part of a sequential combination regimen that

includes T cell directed agents such as cyclosporine, corticosteroids, mycophenolate mofetil, with or without an anti-IL2 receptor antibody. Hence, the anti-CD20 antibody would be considered part of an induction regimen, to be used in conjunction with chronic immunosuppressive therapies. The anti-CD20 antibody may contribute to prevention of an  
5 allorejection response by inhibiting alloantibody production and/or affecting alloantigen presentation through depletion of antigen-presenting cells.

The treatment regimen may entail four weekly infusions (375mg/m<sup>2</sup>) of RITUXAN® administered prior to, or around, transplant. Suitable dosages of the further immunosuppressive agents are as follows: cyclosporine (5mg/kg/day); corticosteroids (1 mg/kg, gradually tapered  
10 off); mycophenolate mofetil (1 gram given twice a day); and anti-IL2 receptor antibody (1mg/kg, five infusions given weekly). The anti-CD20 antibody may also be combined with other induction immunosuppressive drugs, such as polyclonal anti-lymphocyte antibodies or monoclonal anti-CD3 antibodies; maintenance immunosuppressive drugs, such as calcineurin inhibitors (*e.g.*, tacrolimus) and antiproliferative agents (such as azathioprine, leflunomide or  
15 sirolimus); or combination regimens that include blockade of T cell costimulation, blockade of T cell adhesion molecules or blockade of T cell accessory molecules.

Aside from prophylaxis of acute rejection, anti-CD20 antibodies may be used to treat acute rejection. Suitable dosages of the anti-CD20 are as described above. The anti-CD20 antibody is optionally combined with an anti-CD3 monoclonal antibody and/or corticosteroids in  
20 the treatment of acute rejection.

Anti-CD20 antibodies may also be used (a) later in the post-transplant period alone, or in combination with other immunosuppressive agents and/or costimulatory blockade, for treatment or prophylaxis of “chronic” allograft rejection; (b) as part of a tolerance-inducing regimen; or (c) in the setting of xenotransplantation.

25

**Example 4**

**Blocking an Immune Response to a Hemophilic Factor**

A patient with hereditary deficiency of Factor VIII has received multiple transfusions of Factor VIII preparation and developed high titers of anti-Factor VIII antibodies. An anti-CD20



What is claimed is:

1. A method of blocking an immune response to a foreign antigen in a mammal, wherein the mammal is not suffering from a malignancy, comprising administering to the mammal a therapeutically effective amount of an antagonist which binds to CD20.
2. The method of claim 1 wherein the antagonist comprises an antibody.
3. The method of claim 1 wherein the foreign antigen comprises a therapeutic agent.
4. The method of claim 1 wherein the foreign antigen is selected from the group consisting of an antibody, a toxin, a gene therapy viral vector, a graft, an infectious agent, and an alloantigen.
5. The method of claim 1 wherein the mammal is human.
6. The method of claim 2 wherein the antibody is not conjugated with a cytotoxic agent.
7. The method of claim 2 wherein the antibody comprises rituximab (RITUXAN®).
8. The method of claim 2 wherein the antibody is conjugated with a cytotoxic agent.
9. The method of claim 8 wherein the cytotoxic agent is a radioactive compound.
10. The method of claim 9 wherein the antibody comprises Y2B8 or <sup>131</sup>I-B1 (BEXXAR™).
11. The method of claim 1 comprising administering the antagonist intravenously.

12. The method of claim 1 comprising administering the antagonist subcutaneously.
13. The method of claim 2 comprising administering a dose of substantially less than 375mg/m<sup>2</sup> of the antibody to the mammal.
14. The method of claim 13 wherein the dose is in the range from about 20mg/m<sup>2</sup> to about 250mg/m<sup>2</sup>.
15. The method of claim 14 wherein the dose is in the range from about 50mg/m<sup>2</sup> to about 200mg/m<sup>2</sup>.
16. The method of claim 2 comprising administering an initial dose of the antibody followed by a subsequent dose, wherein the mg/m<sup>2</sup> dose of the antibody in the subsequent dose exceeds the mg/m<sup>2</sup> dose of the antibody in the initial dose.
17. The method of claim 4 wherein the foreign antigen is an antibody.
18. The method of claim 17 wherein the antibody is a murine antibody.
19. The method of claim 4 wherein the foreign antigen is a gene therapy viral vector.
20. The method of claim 4 wherein the foreign antigen is a graft.
21. The method of claim 4 wherein the foreign antigen is an alloantigen.
22. The method of claim 1 comprising administering the antagonist to the mammal before the mammal is exposed to the foreign antigen.



23. The method of claim 22 wherein the foreign antigen comprises a graft.
24. A method of treating a mammal comprising administering a therapeutic agent,  
other than an antagonist which binds to CD20, to the mammal and further  
comprising administering an antagonist which binds to CD20 to the mammal,  
wherein the therapeutic agent is immunogenic in the mammal and the antagonist  
blocks an immune response to the therapeutic agent in the mammal.
25. The method of claim 24 comprising administering the therapeutic agent and the  
antagonist essentially simultaneously to the mammal.
26. The method of claim 24 comprising administering the antagonist to the mammal  
prior to the therapeutic agent.
27. The method of claim 24 comprising administering the therapeutic agent to the  
mammal prior to the antagonist.
28. A method of treating graft-versus-host or host-versus-graft disease in a mammal  
comprising administering to the mammal a therapeutically effective amount of an  
antagonist which binds to CD20.
29. A method of desensitizing a mammal awaiting transplantation comprising  
administering to the mammal a therapeutically effective amount of an antagonist  
which binds to CD20.
30. An article of manufacture comprising a container and a composition contained  
therein, wherein the composition comprises an antagonist which binds to CD20,  
and further comprising a package insert instructing the user of the composition to  
treat a patient who has been or will be exposed to a foreign antigen.



[illegible]

## 5

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**COMBINED DECLARATION FOR PATENT APPLICATION  
AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**BLOCKING IMMUNE RESPONSE TO A FOREIGN ANTIGEN**

the specification of which (check one) ☐ is attached hereto or ☒ was filed on July 10, 2000 as Application Serial No. and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):	<div style="border-bottom: 1px solid black; display: inline-block;">Priority Claimed</div>
	<div style="display: inline-block; width: 45%; border-bottom: 1px solid black; text-align: center;">Yes</div> <div style="display: inline-block; width: 45%; border-bottom: 1px solid black; text-align: center;">No</div>

Number	Country	Day/Month/Year Filed
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I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below:

60/144,405	July 16, 1999
Application Ser. No.	Filing Date

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
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**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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